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Insulin-degrading enzyme antagonizes insulin-dependent tissue growth and Aβ-induced neurotoxicity in *Drosophila*

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ABSTRACT

Insulin-degrading enzyme (IDE) is implicated in the pathogenesis of type 2 diabetes mellitus (DM2) and Alzheimer's disease (AD). Here we provide genetic evidence that *Drosophila Ide* (*dIde*) antagonizes the insulin signaling pathway and human Aβ-induced neurotoxicity in *Drosophila*. In this study, we also generated a *dIde* knockout mutant (*dIde*^{KO}) by gene targeting, and found that loss of IDE increases the content of the major insect blood sugar, trehalose, thus suggesting a conserved role of IDE in sugar metabolism. Using *dIde*^{KO} as a model, further investigations into the biological functions of IDE and its role in the pathogenesis of DM2 and AD can be made.

1. Introduction

Insulin is an evolutionally conserved peptide hormone that is secreted from the pancreas and promotes glucose uptake in muscle and adipose tissue. Insulin also stimulates cell growth and differentiation, and promotes the storage of glucose and lipids by stimulating amino acid uptake, protein synthesis, glycogenesis, and lipogenesis [1]. On the other hand, insulin inhibits gluconeogenesis in the liver, which reduces blood glucose concentration. Patients with type 2 diabetes mellitus (DM2) show insulin resistance and defects in insulin secretion from the pancreas [2]. Although the pathogenic mechanism of DM2 is presently unclear, insulin resistance is thought to be an essential causative factor [3].

Alzheimer's disease (AD) is often associated with DM2 [4]. The risk of developing AD increases 1.6-fold in patients with DM2 [5]. Brains of AD patients exhibit neurodegeneration, neuronal cell death, and tissue atrophies. It has been suggested that AD-linked neurodegeneration is caused by an accumulation of β-amyloid peptide (Aβ) [6]. Both amyloid plaque and soluble Aβ oligomers are thought to have neurotoxic effects that promote neurofibrillary tangle formation [7]. In patients with late-onset AD (LOAD), peptide clearance can be impaired, which results in cerebral Aβ accumulation [8].

Insulin-degrading enzyme (IDE), a zinc metalloendopeptidase, has been identified as a candidate gene for diabetes susceptibility in the Goto-Kakizaki (GK) rat, a genetic model of non-insulin-dependent diabetes [9]. The GK allele of IDE has been regarded as a partial loss-of-function mutation, since it has two amino acid substitutions that reduce insulin-degrading activity by 31% [9]. In humans, genome-wide association studies reveal that the IDE region of chromosome 10q contains a variant that confers DM2 risk [10]. Interestingly, four markers near or in the IDE locus are associated with AD in LOAD families [11], and catalytic activity and expression of IDE are found to be reduced in LOAD patients [12]. Indeed, deletion of IDE in mice has been shown to cause accumulation of cerebral Aβ, hyperinsulinemia, and glucose intolerance [13]. Furthermore, impaired Aβ catabolism has also been observed in primary cultured fibroblasts and neurons derived from GK rats [14]. These studies strongly support the notion that IDE is involved in the pathogenesis of both DM2 and AD.

IDE is highly conserved from yeast to human. *Drosophila* IDE shows 44% identity and 59% similarity in amino acid sequence to that of human IDE (hIDE). *Drosophila Ide* (*dIde*) has physical and kinetic properties very similar to those of hIDE, including molecular weight, optimal pH for activity, and the ability to degrade insulin in vitro [15,16]. However, it is not clear whether *dIde* regulates the insulin signal and AD-related phenotypes in vivo. Here, we

Abbreviations: IDE, insulin-degrading enzyme; DM2, type 2 diabetes mellitus; AD, Alzheimer's disease; LOAD, late-onset AD; GK rat, Goto-Kakizaki rat; *dilp2*, Drosophila insulin-like peptide 2; InR, insulin receptor; Aβ, β-amyloid; APP, Aβ precursor protein; BACE, β-site APP-cleaving enzyme; AICD, APP intracellular domain

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provide genetic evidence that *dIde* antagonizes *Drosophila insulin-like peptide 2* (*dilp2*) signaling and the neurotoxicity that is induced by ectopically expressed human Aβ in *Drosophila*. In addition, we generated a *dIde* null mutant and found that *dIde* is involved in blood sugar homeostasis, suggesting that the function of IDE is conserved between mammals and *Drosophila*.

2. Materials and methods

2.1. Fly strains

y w^{67c23} *Df(1)*w^{67c23} was used as a recipient for transgenic flies. *elav-Gal4*, *GMR-Gal4*, *sd-Gal4*, and *UAS-InR* were obtained from Bloomington Stock Center. *Act5C-GAL4* and *UAS-mCD8::GFP* were from Kyoto Stock Center. *dilp2-Gal4* [17] and *UAS-dilp2* [18] were gifts from Dr. E. Hafen. *P{70FLP} P{70I-SceI}* and *P{70FLP}*10 were gifts from Dr. J.J. Sekelsky [19]. *UAS-APP* and *UAS-BACE* were gifts from Dr. R. Reifegerste [20]. *PTEN* was a gift from Dr. D. Pan [21]. Transgenic flies carrying *UAS-dIde*, *UAS-hIDE*, were generated by *P*-element-mediated transformation [22]. cDNAs corresponding to the coding regions of *dIde* and *hIDE* were obtained by RT-PCR using mRNAs isolated from adult *Drosophila* and human HEK293 cells, respectively, and subcloned into the pUAST vector [23]. Gene targeting was performed as described previously [24,25]. For constructing the *P{donor}* plasmid for gene targeting, approximately 3 kb of both upstream and downstream regions of the *Ide* locus were amplified by PCR with the following primers: *ide*Tg55*XhoI*: 5′-CTCGAGTCTGAAAACGCCTGATCCC-3′, *ide*Tg53*NheI*: 5′-GCTAGC-GCGCCAGTTTCAGGTTCT-3′, *ide*Tg35*NotI*: 5′-GCGGCCGCGAGAAAC-GTTTTGACATCC-3′, and *ide*Tg33*SmaI*: 5′-CCCGGGAACTTAGATACTGGCTATGCG-3′. The sequences of PCR-amplified fragments were confirmed, and subcloned into the polylinker of p{EndsOut2} (a gift from J.J. Sekelsky). The *mini-white* gene was inserted between the upstream and downstream target sequences as a marker for transformation. Targeted lines were backcrossed to the *y* w^{67c23} *Df(1)*w^{67c23} stock for six generations.

2.2. Measurement of body weight and wing size

Flies were weighed using an Analytical Semi-Micro Balance (A&D Company, Tokyo, Japan). To measure wing size, the wings were mounted in a drop of FLY LINE DRESSING (TIEMCO, Tokyo, Japan). Wing images were captured using a Leica MZ16F stereoscopic microscope equipped with a CCD camera. Wing area was measured using the Object-Image Program (Image J 1.41).

2.3. Cryostat section

Fly heads were fixed in 2% formaldehyde/PBT (0.2% Tween-20) for 120 min at 4 °C. After washing in PBT, the tissues were incubated in 12% sucrose/PBT overnight at 4 °C. The tissues were immersed in a drop of O.C.T. Tissue Tek Compound (Miles Inc., Elkhart, IN, USA) in 96 well plates, and frozen in liquid nitrogen. The samples were cut into 10-μm sections using a cryostat. The sections were immediately fixed in 0.5% formaldehyde/PBS for 60 min at RT. The slides were washed in PBS and incubated with rhodamine-phalloidin (Invitrogen)/PBS (1/1000) overnight at 4 °C in a moist chamber. The samples were washed in PBS, mounted in GEL/MOUNT (Cosmo Bio, Tokyo, Japan), and observed under a fluorescence microscope (Olympus BX50).

2.4. Longevity

Longevity was determined as described previously [26]. Newly eclosed flies were collected within 24 h, they were transferred every 2–3 days into fresh food vials until all flies had died, and the number of dead flies was counted at the time of each transfer. Twenty flies were kept in a glass vial at 25 °C and at least 100 flies were used for each genotype.

2.5. Glucose and trehalose measurement

Hemolymph was collected from either 25 female or 40 male flies. Flies were punctured in the thorax with a fine needle and put into 0.5-ml tubes whose bottoms had been punctured with a 22-gauge needle (Terumo, Tokyo, Japan) and plugged with cotton. The tubes were set into 1.5-ml tubes and centrifuged at 2600×*g* for 5 min at 4 °C. The hemolymph was heated at 95 °C for 5 min. Glucose concentration was measured using a Glucose Assay Kit (Sigma). Trehalose in the hemolymph was determined after digesting in glucose and incubating with 0.5 U trehalase (Sigma) at 37 °C for 12 h.

3. Results and discussion

3.1. Overexpression of dIde or hIDE antagonizes insulin-dependent tissue growth

To investigate the function of *Drosophila Ide* (*dIde*), we generated transgenic flies bearing *UAS-dIde* that is induced upon GAL4

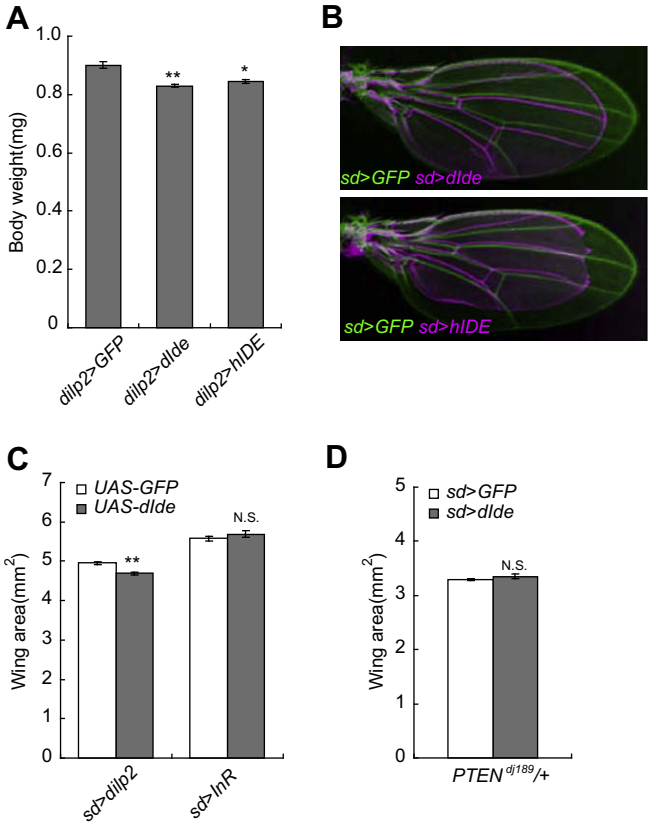

Fig. 1. Functional relationship between IDE and the insulin signaling pathway. Body weight of flies overexpressing *dIde*, hIDE, and GFP (control) using *dilp2-GAL4* (A). Effects of forced expression of *dIde* (upper panel) or *hIDE* (lower panel) on wing size (B). *sd-GAL4* was used as a driver. Superimposed images of the wings overexpressing IDE (magenta) and the control wings expressing GFP (green). Genetic interaction between *dIde* and the components of the insulin pathway. *dIde* or GFP were ectopically expressed together with *dilp2* or *InR* in developing wing imaginal discs using *sd-GAL4* (C), or in combination with the *PTEN* mutation (D). Bars indicate the mean body weight or the mean wing size ±S.E. The *t*-test was used to evaluate the differences in means between two groups (NS: not significant, $^{*}P < 0.05$, and $^{**}P < 0.001$).

activation. We also generated *UAS-human IDE* (*hIDE*) transgenic flies to compare the functions with those bearing *UAS-dIde*. Since insulin promotes protein synthesis, cell growth and proliferation, we examined the effects of *dIde* overexpression on body size. When *dIde* or *hIDE* were misexpressed in insulin producing cells using *dilp2-GAL4* as a driver, the body weight of the adult flies was reduced to 92% and 94% of control flies, respectively (Fig. 1A). We also tested wing size, which is suitable for quantitative analysis of tissue growth. Forced expression of either *dIde* or *hIDE* was induced in the developing wing imaginal discs using *sd-GAL4*. We found that wing size was significantly reduced in these flies: 82% and 83% of the control, respectively (Fig. 1B). These results indicate that both *dIde* and *hIDE* negatively regulate tissue growth.

Next we investigated the functional relationship between *dIde* and the insulin signaling pathway more specifically. *Drosophila insulin-like peptide 2* (*dilp2*) promotes tissue growth [18]. Misexpression of *dilp2* in developing wing imaginal discs produces adult flies with a wing size larger than that of control. We found that this phenotype is suppressed significantly when *dilp2* is co-expressed with *dIde*, suggesting that *dIde* inhibits *dilp2*-induced growth (Fig. 1C). *dilp2* promotes growth through the *insulin receptor* (*InR*) [18]. Overexpression of *InR* in the wing imaginal disc also increases wing size. However, unlike those induced by *dilp2*, the *InR*-induced phenotype is not suppressed by co-overexpression of *dIde*, suggesting that *dIde* acts upstream of *InR* (Fig. 1C). We also determined the genetic interaction between *dIde* and *PTEN*, which functions downstream of *InR* to negatively regulate the insulin signal by inhibiting PI3K activity. Loss-of-function mutations in *PTEN* increase body size by elevating PI3K activity [27]. We found that overexpression of *dIde* had no effect on the large wing phenotype caused by the *PTEN* mutation (Fig. 1D). These genetic experiments strongly suggest that *dIde* negatively regulates the insulin signaling pathway, most likely by functioning between the *Dilp2* ligand and InR.

3.2. Overexpression of dIde or hIDE reduces Aβ-induced neurotoxicity

It has been shown that human IDE is capable of digesting both β-amyloid (Aβ) and the Aβ precursor protein (APP) intracellular domain (AICD) in vitro. To examine whether overexpression of *dIde* can suppress the neurotoxicity induced by Aβ in vivo, we used the *Drosophila* AD model, in which human APP and the β-site APP-cleaving enzyme (BACE) are misexpressed in photoreceptor neurons [20]. In this model, a highly organized architecture of retinal photoreceptors degenerates in an age-dependent manner (Fig. 2A). We found that forced expression of *dIde* or *hIDE* can suppress neuronal degeneration in this model. It has been reported that ubiquitous overexpression of APP and BACE using *Act5C-GAL4* causes ectopic wing vein formation [20]. In this study, the ectopic wing vein phenotype was also suppressed by forced expression of either *dIde* or *hIDE* (Fig. 2B). In addition, we found that pan-neural overexpression of APP and BACE using *elav-GAL4* shortens the longevity of adult flies: the average lifespan of *elav > APP BACE* and control flies (*elav > GFP*) was 49.3 ± 1.2 and 58.9 ± 1.3 days, respectively (Fig. 2C and D). The reduced lifespan was partially rescued by forced expression of *dIde* or *hIDE*: the average lifespan was 52.9 ± 1.2 and 54.4 ± 3.0 days, respectively (Fig. 2C and D). These results suggest that *dIde* or *hIDE* can inhibit the pathological processes associated with Aβ and/or AICD accumulation in vivo. Taken together with these findings, the *Drosophila* AD model might be useful to evaluate single nucleotide polymorphisms (SNPs) within the coding regions of APP and IDE in humans.

3.3. Generation and phenotypic characterization of the dIde knockout mutant

To gain insight into the role of *dIde* in *Drosophila*, we generated a *dIde* knockout mutant ($dIde^{KO}$) by gene targeting. In this mutant,

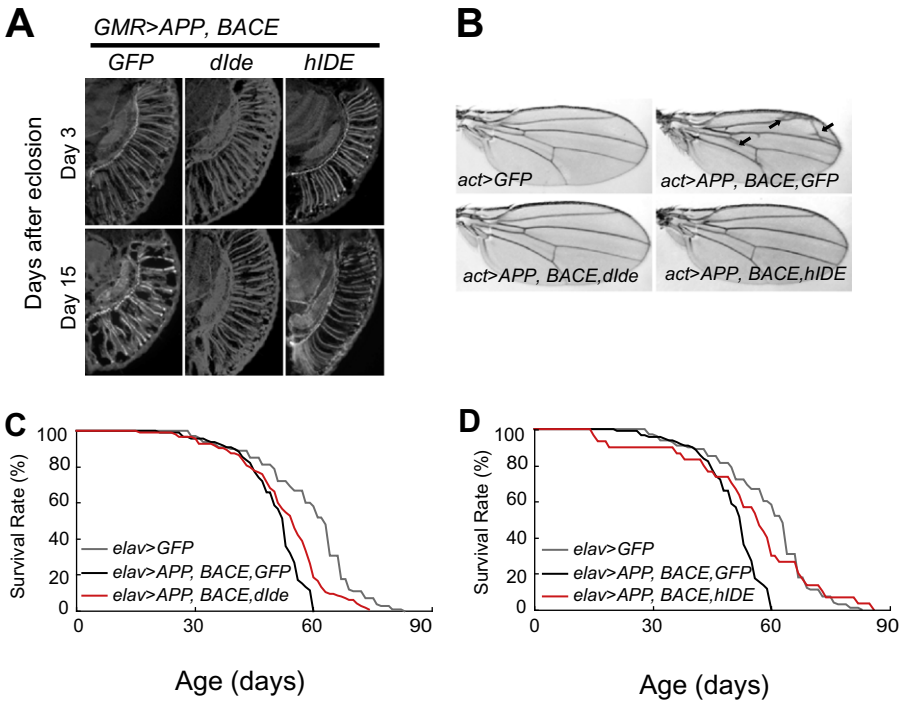

Fig. 2. Overexpression of IDE in the *Drosophila* AD model. Cryostat sections of photoreceptor neurons in AD model flies aged to day 3 (upper panels) and 15 (lower panels) (A). Human APP and BACE were ectopically expressed in *Drosophila* photoreceptor neurons using GMR-GAL4. GFP (control, left), *dIde* (middle), and *hIDE* (right) were co-expressed in the AD model. Effects of IDE overexpression on extra vein formation in AD model flies expressing APP and BACE ubiquitously under control of *act-GAL4* (B). Forced expression of *dIde* (lower left) or *hIDE* (lower right) suppresses extra vein formation (upper right). Arrows indicate extra veins. Survival curves of male flies overexpressing GFP in wild type (gray) and the AD model (black), and those overexpressing *dIde* (C) or *hIDE* (D) in the AD model (red). Co-expression of *dIde* and *hIDE* extends mean longevity compared to the control, which expresses GFP by 7% and 10%, respectively. The log lank test was used to evaluate the difference between two survival curves ($P < 0.0001$).

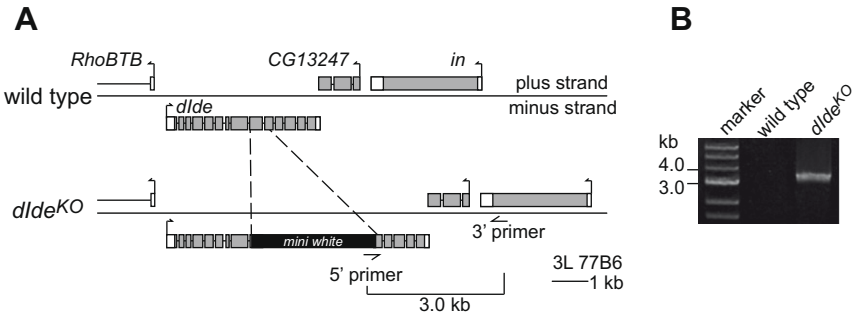

Fig. 3. Generation of *dIde* knockout flies by gene targeting. Genomic organization of the *dIde* locus and the targeted mutant allele (A). Gray and open boxes represent exons encoding the protein-coding and untranslated regions, respectively. Arrows with gene names indicate the transcription start sites and the direction. Confirmation of targeting events (B). Targeting events were confirmed by genomic PCR using the indicated primers. A 3-kb fragment was amplified from genomic DNA of the mutant allele ($dIde^{KO}$), but not from that of the wild type.

the *Drosophila mini-white* gene replaces part of its genomic region (exons 9 and 10) (Fig. 3A). The targeting event was confirmed by genomic PCR analysis (Fig. 3B). Since mutated *dIde* lacks its substrate-binding domains, $dIde^{KO}$ flies are thought to be null. Flies homozygous for $dIde^{KO}$ were found to be viable and fertile with no obvious developmental defects. No significant difference was observed in mean body weight between $dIde^{KO}$ and wild type (Fig. 4A). In mice, knockout of IDE results in hyperinsulinemia, chronic elevation of insulin due to the reduction of insulin-degradation, and glucose intolerance, which are all hallmarks of DM2 [13]. To investigate whether $dIde^{KO}$ flies have defects in glucose homeostasis, we measured the concentration of glucose and trehalose, the insect blood sugar, in the hemolymph of $dIde^{KO}$ flies. We found no significant difference in glucose concentration between $dIde^{KO}$ and wild type, but the trehalose level in $dIde^{KO}$ flies increased by 86% compared to that of the wild type (Fig. 4B). We measured the longevity of $dIde^{KO}$ flies, and found that the mutant flies live longer (56.2 ± 1.2 and 63.8 ± 0.6 days for males and females, respectively) when compared to wild type (53.2 ± 1.0 days for males and 57.3 ± 1.0 days for females) (Fig. 4C). The mechanism of longevity extension by $dIde^{KO}$ is currently unknown, but *dIde* might affect the regulation of energy metabolism. Although the majority of insulin degradation by IDE takes place in endosomes and cytosol, and at the plasma membrane, IDE is also found in mitochondria [15,28]. IDE that is localized in mitochondria appears to bind SIRT4, which inactivates glutamate dehydrogenase (GDH) through ADP-ribosylation [29]. Since GDH catalyzes the conversion of glutamate to α-ketoglutarate to fuel the tricarboxylic acid cycle, the absence of IDE might result in an alteration of energy supply from the amino acids. The function of IDE other than insulin-degradation is not well understood. *Drosophila dIde* knockout mutants are a useful model system to further investigate the role of IDE in the regulation of longevity and the pathological process of AD and DM2.

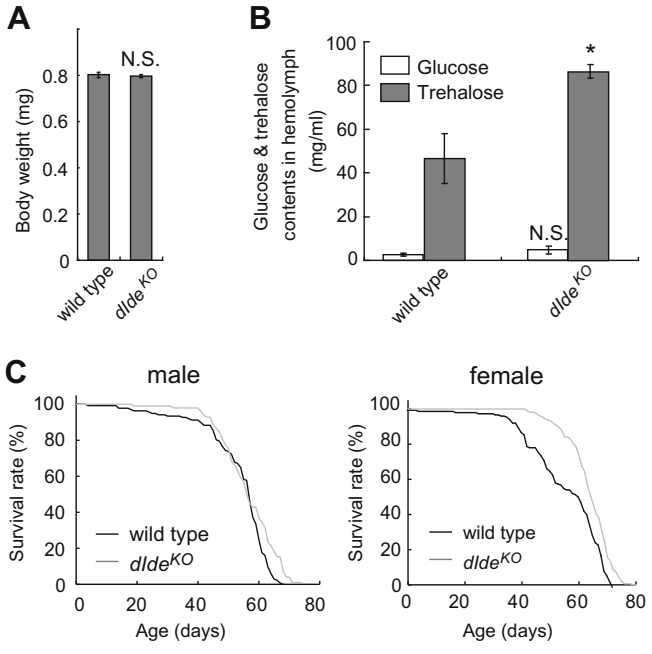

Fig. 4. Phenotypic characterization of the *dIde* knockout mutant. Comparison of body weight (A) and blood sugar content (B) between $dIde^{KO}$ and wild type flies. Bars indicate the mean values (±S.E.) of body weight (A) and each sugar content (B). Statistical significance (*t*-test): NS: not significant, $^{*}P < 0.01$. Survival curves of $dIde^{KO}$ and wild type flies (C). $dIde^{KO}$ flies live longer than wild type flies for both sexes (log lank test, $P < 0.001$).

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